Exhibit 2

Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter

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ABSTRACT

Gene-targeted mice derived from embryonic stem cells are a useful tool to study gene function during development. However, if the mutation is embryonic lethal and the gene is deleted from the onset of development, later functions in adult animals cannot be studied. Recently, the bacterial Cre-loxP site-specific recombination system has successfully been used in transgenic animals to produce tissue-specific and temporal deletions [Gu *et al.* (1993) *Cell*, 73, 1155–1164; Gu *et al.* (1994) *Science*, 265, 103–106; Kühn *et al.* (1995) *Science*, 269, 1427–1429]. We have evaluated the tetracycline responsive binary system [Gossen and Bujard (1992) *Proc. Natl. Acad. Sci. USA*, 89, 5547–5551] for its ability to transiently express the Cre recombinase in transgenic mice. In this system, a transactivator fusion protein composed of the tetracycline repressor (tetR) and the acidic domain of the herpes simplex viral protein 16 (VP16) can regulate the expression of the Cre gene from a promoter containing tet-operator (tetO) sequences. In the absence of tetracycline, the Cre gene is expressed and will induce site-specific recombination between two loxP sites. In the presence of tetracycline, the Cre gene will not be expressed and recombination will not occur.

We generated two independent transgenic mouse lines that carry the *tetR/VP16* transactivator gene under the control of the human cytomegalovirus immediate early gene 1 promoter-enhancer (CMV-tTA; described in 1,2) and a reporter construct in which the CMV promoter and a nuclear β-*galactosidase* gene are separated by transcriptional Stop sequences (described in 3) flanked by two *loxP* sites (*loxP*–β*gal*; Fig. 1). Both lines were mated to mice containing the *Cre* gene under the control of a basal CMV promoter fused to seven copies of the tetO sequences (tetO-Cre). Recombination can therefore be detected in the offspring by β-galactosidase activity resulting from Cre mediated deletion of the Stop sequences.

In the absence of tetracycline, β-galactosidase staining was observed in animals containing all three transgenes (Fig. 2A).
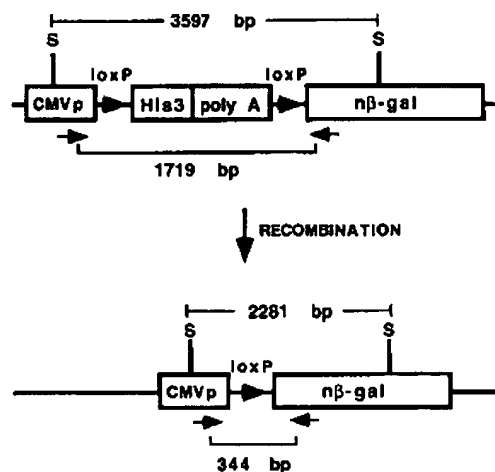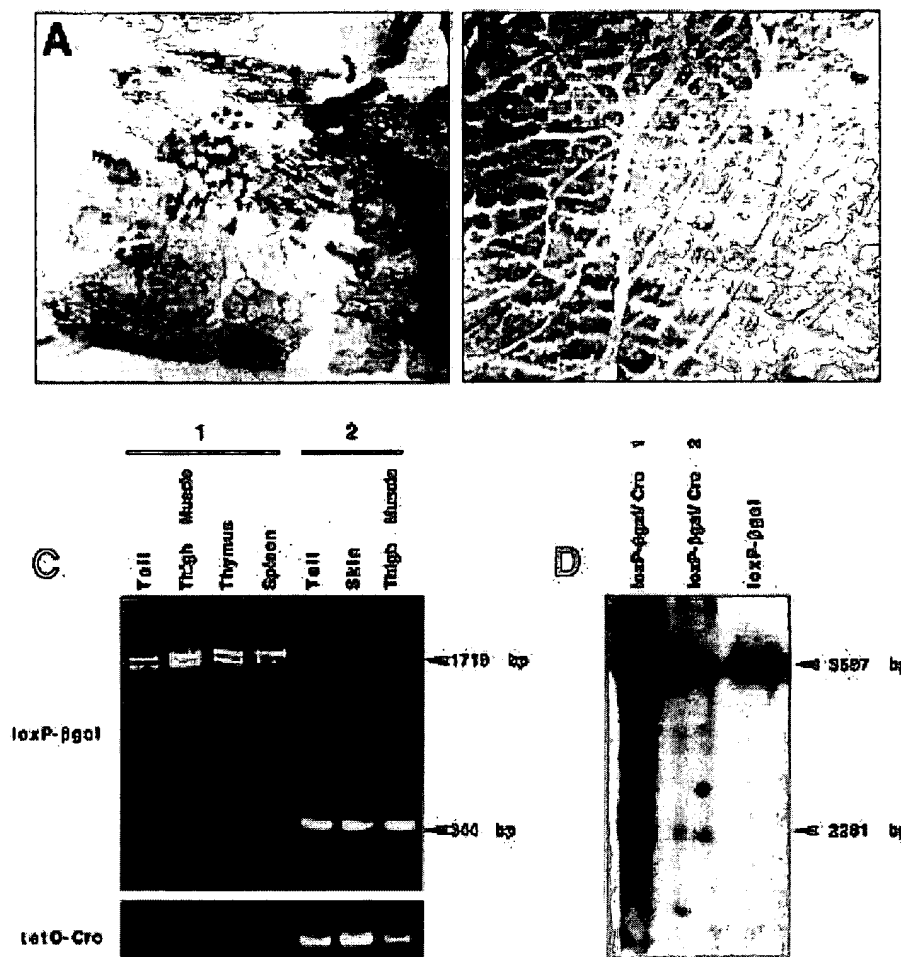

Figure 1. Structure of the *loxP*-β*gal* transgene used for verifying recombination in transgenic mice. The human cytomegalovirus immediate early gene 1 promoter-enhancer (CMV) and a nuclear β-*galactosidase* gene (nβ-gal) are separated by transcriptional Stop sequences flanked by two *loxP* sites. The Stop sequences consist of a C-terminal sequence of yeast *His3* gene, an SV40 polyadenylation signal (polyA) and a 5' splice donor site. Recombination between the *loxP* sites will delete the Stop sequences and induce β-*galactosidase* expression. Arrows represent the primers used in PCR analysis of recombination events. Abbreviation: S, SacI.

β-galactosidase activity was detected in both loxP-βgal lines. Since Cre expression is dependent on *tetR/VP16* transactivator expression, the efficiency of recombination is relative and varies according to CMV-tTA activity. Indeed, staining levels in triple transgenic mice were similar to those observed when the β-*galactosidase* gene was under direct control of CMV-tTA (2) and can be attributed to the variability and mosaicism associated with the CMV promoter (4). In control experiments, we observed no β-galactosidase activity in mice lacking the *tetO-Cre* transgene (Fig. 2B). Recombination was also detected by PCR analysis using primers located in the CMV promoter and the β-*galactosidase* gene. (Primers for the *CMV-tTA* and *loxP*-β*gal* transgene were described in ref. 2. Primers for the *tetO-Cre* transgene are 5'-CCA TGC CGC CCA CGA CCG GC-3' and

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Figure 2. (A) β-galactosidase activity in thigh muscle tissue sections from an adult mouse carrying all three transgenes. Cells with black nuclei expressed the recombined β-*galactosidase* gene. (B) β-galactosidase activity in thigh muscle tissue sections from an adult mouse carrying only the *CMV-tTA* and *loxP-βgal* transgenes. (C) PCR analysis of DNA isolated from different tissues of two adult transgenic mice. The mouse that did not carry the *Cre* transgene (No. 1) produced two fragments: a 1719 bp fragment corresponding to the nonrecombined *loxP-βgal* transgene and a slightly smaller fragment that arise from one of the primers annealing to a sequence within the Stop region. The mouse containing a Cre transgene (No. 2) generated a 344 pb recombined fragment. (D) Southern blot analysis of recombination in transgenic mice. Non-recombined *loxP-βgal* transgene produces a 3597 bp *Sac*I fragment while the 2281 bp recombined fragment is only detected in mice containing the *Cre* transgene.

5'-CGA GCT CGG TAC CCG GGC CGA-3'.) In animals without the *tetO-Cre* transgene, a 1719 bp non-recombined fragment was observed while animals containing the *Cre* gene generated a 344 bp recombined fragment (Fig. 2C). Recombination could be detected in different tissues of an animal. Note that absence of the 1719 bp fragment does not indicate that recombination occurred in every cell since non-recombined transgenes were detected in Southern blot analysis (Fig. 2D). The absence of the non-recombined fragment is due to the fact that PCR kinetics favors amplification of the shorter target sequence. These results demonstrate that the tetR/VP16 transactivator can activate expression of Cre recombinase which in turn induce recombination between the *loxP* sites.

To verify if recombination could be repressed, slow-release tetracycline pellets (Innovative Research of America) were implanted subcutaneously into female mice transgenic for both

the *CMV-tTA* and *loxP-βgal* transgenes. The pellets released 1 mg tetracycline hydrochloride per day. Seven days after implantation, loxP-βgal mice were mated to male tetO-Cre mice and 31 newborn animals carrying all three transgenes were analyzed by PCR for recombination. No recombination was detected in different tissues of half of the newborns (Fig. 3A) while varying levels of recombination could be observed in others (Fig. 3B). Leakiness varied between litters and in some cases, between littermates. In some litters, recombination was repressed in all offsprings while recombination could be detected in some newborns of a same litter. This leakiness may be due to insufficient or fluctuating concentrations of tetracycline within a pregnant animal during gestation resulting in sporadic Cre expression in the embryos. It is also possible that in some newborns, basal levels of *Cre* expression are sufficient to induce recombination. Recently, a mutant tetR/VP16 that binds and
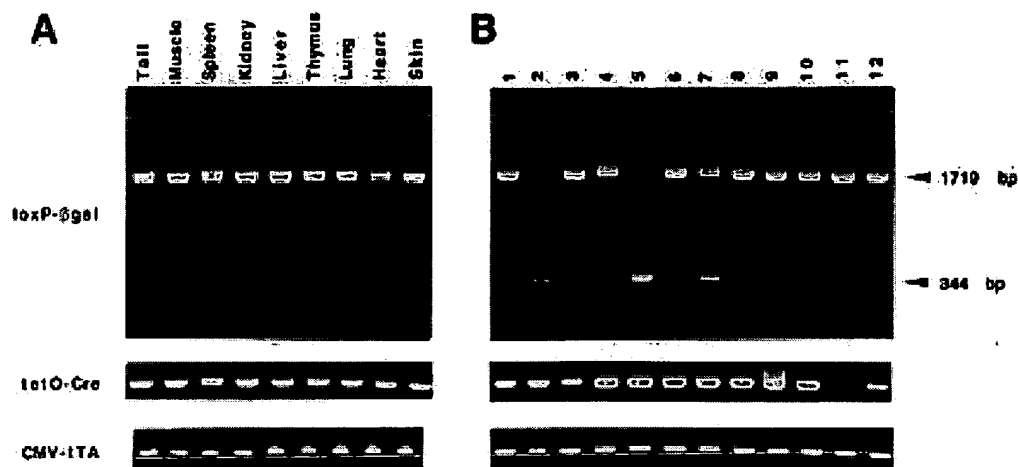
Figure 3. PCR analysis of newborn mice in which the mother was treated with tetracycline. (A) Analysis of DNA isolated from different tissues of a newborn mouse carrying all three transgenes. Only the 1719 bp non-recombined loxP-βgal fragment was observed. (B) Analysis of tail DNA isolated from various newborn mice. In some mice, the 344 bp recombined fragment was detected.

transactivates the tetO-promoter in the presence of tetracycline was developed (5). This mutant transactivator may permit a better control of *Cre* expression since tetracycline would be administered to activate expression instead of repressing it.

ACKNOWLEDGEMENTS

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